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MECHANISMS OF ACTION: PHYSIOLOGICAL EFFECTS

In Vitro and *In Vivo* Antibiotic Capacity of Two Host Defense Peptides

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ABSTRACT Two nonamidated host defense peptides named Pin2[G] and FA1 were evaluated against three types of pathogenic bacteria: two (*Staphylococcus aureus* UPD13 and *Pseudomonas aeruginosa* UPD3) isolated from diabetic foot ulcer patients, and another (*Salmonella enterica* serovar Typhimurium [ATCC 14028]) from a commercial collection. *In vitro* experiments showed that the antimicrobial performance of the synthetic peptides Pin2[G] and FA1 was modest, although FA1 was more effective than Pin2[G]. In contrast, Pin2[G] had superior *in vivo* anti-infective activity to FA1 in rabbit wound infections by the diabetic foot ulcer pathogens *S. aureus* UPD13 and *P. aeruginosa* UPD3. Indeed, Pin2[G] reduced bacterial colony counts of both *S. aureus* UPD13 and *P. aeruginosa* UPD3 by >100,000-fold after 48 to 72 h on skin wounds of infected rabbits, while in similar infected wounds, FA1 had no major effects at 72 to 96 h of treatment. Ceftriaxone was equally effective versus *Pseudomonas* but less effective versus *S. aureus* infections. Additionally, the two peptides were evaluated in mice against intragastrically inoculated *S. enterica* serovar Typhimurium (ATCC 14028). Only Pin2[G] at 0.56 mg/kg was effective in reducing systemic (liver) infection by >67-fold, equivalent to the effect of treatment with levofloxacin. Pin2[G] showed superior immunomodulatory activity in increasing chemokine production by a human bronchial cell line and suppressing polyinosinic-polycytidylic acid (poly[I:C])-induced proinflammatory IL-6 production. These data showed that the *in vitro* antimicrobial activity of these peptides was not correlated with their *in vivo* anti-infective activity and suggest that other factors such as immunomodulatory activity were more important.

KEYWORDS antibiotic, antimicrobial peptide, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, peptide, *Pseudomonas aeruginosa*, host defense peptide

Antimicrobial resistance has emerged as one of the principal public health problems of the 21st century, threatening the effective cost prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses, and fungi no longer susceptible to the common medicines used to treat them (1). Emerging antibiotic resistance among *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella* spp., among other bacteria, is problematic, because these organisms are common causes of a variety of nosocomial, community acquired, and skin and soft tissue infections, e.g., diabetic foot ulcers and gastrointestinal infections. Foot ulcers in type 2 diabetes mellitus patients have a lifetime incidence of 19 to 34% and become infected in 40 to 80% of cases (2). This can lead to the spread of infection to soft tissues, and eventually to bone, which is a major cause of lower limb amputations. Diabetic foot infections are caused mainly by pathogens such as *S. aureus*, *Streptococcus pyogenes*,

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TABLE 1 MICs and hemolytic activity *in vitro* for antibiotics, Pin2[G], and FA1

Antibiotic	MIC (μg/ml)					Hemolytic activity IC_{50} (μg/ml)[b]
	S. aureus		*P. aeruginosa*		*S. enterica* serovar Typhimurium	
	ATCC 25923	UPD13	ATCC 27853	UPD3	ATCC 14028	
Azithromycin	1.5	>50	12.5	50	3.5	nd
Ceftriaxone	1.5	>50	12.5	25	1.5	nd
Vancomycin	1.5	1.5	>50	>50	50	nd
Sulfamethoxazole	>50	>50	>50	>50	25	nd
Trimethoprim	1.5	1.5	>50	>50	1.5	nd
Levofloxacin	1.5	>50	1.5	1.5	1.5	nd
Amoxicillin	1.5	50	>50	>50	1.5	nd
Gentamicin	1.5	1.5	1.5	1.5	1.5	nd
Pin2[G]	12.5[a]	7.5	>50[a]	60	150	1.2
FA1	>50	>250	25	50	35	>250

[a]From Arenas et al. (15).
[b]Release of hemoglobin was quantified as indicative of cell lysis; 2.3×10^8 erythrocytes/ml were incubated at 37°C for 1 h. nd, not determined.

the family *Enterobacteriaceae*, and anaerobes, while other species are also present, including *Pseudomonas aeruginosa* and *Stenotrophomonas*, can be important (3). Significantly, for several of these common pathogens, antibiotic resistance occurs due to the treatment of recurrent and polymicrobial biofilm infections in individual diabetic patients (4).

In a related vein, gastrointestinal infections are also a major worldwide public health concern. For example, Salmonellosis is caused by *Salmonella enterica* serotypes and is typically characterized by self-limiting gastroenteritis, manifested as diarrhea, fever, and abdominal pain (5), with particular issues in infants, young children, the elderly, and the immunocompromised (6). The main reservoir of nontyphoidal *Salmonella* is the intestinal tract of food-producing animals, and foodborne organisms are the most relevant source with high impact on human health (7). Strains have been reported in some countries with resistance to older antibiotics such as chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole (5, 8).

Here, we tested the *in vitro* and *in vivo* efficacy of two synthetic venom-derived host defense peptides (HDPs), Pin2[G] and FA1, to observe their efficacy. We utilized topical infection models in rabbits with selected *S. aureus* and *P. aeruginosa* strains from diabetic foot ulcer patients. Likewise, we implemented a model of intragastric infection in mice with *Salmonella* serovar Typhimurium ATCC 14028. Overall the data indicate that the *in vivo* efficacy of our peptides was not related to their *in vitro* activity, but rather that their immunomodulatory activity might be more important.

RESULTS

Antimicrobial activity of clinical isolates. *S. aureus* UPD13 and *P. aeruginosa* UPD3 were tested against eight commercial antibiotics with different mechanisms of action (levofloxacin, trimethoprim, sulfamethoxazole, azithromycin, amoxicillin, ceftriaxone, gentamicin, and vancomycin; see Table 1). Results were compared to that of the reference strains *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853. The strain *S. aureus* UPD13 was more resistant to five antibiotics compared to *S. aureus* strain ATCC 25923 (Table 1). *P. aeruginosa* strain UPD3 was resistant to the same antibiotics as *P. aeruginosa* ATCC 27853 (Table 1).

Bacterial strains were also tested against the HDPs Pin2[G] and FA1. The MICs of Pin2[G] against *S. aureus* UPD13, *P. aeruginosa* UPD3, and *S.* Typhimurium ATCC 14028 were quite high, with moderate activity (MIC = 7.5 μg/ml) only against the Gram-positive organism *S. aureus*. In contrast, FA1 had very weak activity with MICs of >250, 50, and 35 μg/ml against these 3 organisms, respectively, and was somewhat better against the Gram-negative organisms (Table 1).

To assess selectivity, the hemolytic activity of Pin2[G] and FA1 was tested against human erythrocytes. Pin2[G] proved to be highly hemolytic in phosphate-buffered saline (PBS),

with a 50% inhibitory concentration (IC_{50}) of 1.2 μg/ml. In contrast, FA1 was nonhemolytic at these concentrations, with an IC_{50} greater than 256 μg/ml (Table 1).

***In vivo* activity in topical infections.** The results showed that Pin2[G] and FA1 lacked acute irritant effects in rabbits. No clinical signs of irritation were observed in the rabbit groups treated with either Pin2[G] or FA1. Also, no dermal responses, including erythema/eschar or edema, and complete hair recovery was observed during the prolonged application at the peptide concentrations used. During the following days the mood and weight of the rabbit was monitored without observing any type of side effect. Since there were no discernible changes in epidermis of rabbits before and after the test, it was decided to explore the anti-infective effect.

Pin2[G] and FA1 were tested in a subcutaneous wound infection model with both *S. aureus* UPD13 and *P. aeruginosa* UPD3. Four wounds/animal were created on the backs of four different New Zealand rabbits. Three wounds/rabbit were infected with either *S. aureus* UPD13 or *P. aeruginosa* UPD3, and one was uninfected and treated with only PBS. One of the infected wounds was treated with ceftriaxone, another with either Pin2[G] or FA1. The third one was infected but treated with only distilled water to serve as a negative control, while a fourth uninfected one received only distilled water. Treatments were administered every 24 h for 4 days, and, prior to treatment, the wound area was swabbed to quantify the number of bacteria present on that day within the wound.

Treatment with Pin2[G] decreased both *S. aureus* UPD13 and *P. aeruginosa* UPD3 infections (Fig. 1A and B), with complete clearance at 48 h for *S. aureus* (a 1.3×10^5 CFU decrease compared to control) and 72 h for *P. aeruginosa* (a 5.2×10^5 CFU decrease compared to control). Ceftriaxone demonstrated equivalent activity versus *P. aeruginosa*, but had no major activity against *S. aureus*. In contrast, as seen *in vitro*, FA1 was unable to decrease bacterial growth of *S. aureus* UPD13 and *P. aeruginosa* UPD3 (Fig. 2A and B). These data show that the *in vitro* MIC experiments did not reflect the *in vivo* observations.

Toxicity in mice. For Pin2[G] and FA1, the results of mice weight for doses 0.28, 5.6, 8.4, 11.2, and 14 mg/kg either for male or female, and the control group at 0, 1, 7, and 14 days passed the normality tests (Fig. 3A and B). It is important to note that at 24 h post administration, all the animals behaved normally without no signs of toxicity; that is, no clinical signs such as mortality, locomotive activity, sensitivity to sound, abnormal tail appearance, abnormal exploratory behavior, aggressive behavior, seizures, muscle tone in extremities, somatic response, prostration, tremors, lacrimation, ptosis, defecation, micturition, changes in respiration, discharges from the nose, or cyanosis were observed.

***In vivo* activity in gastric infection.** Five groups of *Salmonella*-free mice ($n = 5$) of 2 weeks of age were orally infected with 10^8 CFU/ml of *S.* Typhimurium. All five groups were treated after 1 day postinfection, every 24 h for a week. The control group was treated with just vehicle solution. After treatment, all five groups were sacrificed and sections from liver were collected and processed for bacterial count. The bacterial count of each group is shown in Table 2, while Fig. 4 shows the geometric means $\pm$ standard errors of the means (five mice per treatment). Treatment with 0.56 mg/kg of Pin2[G] reduced the bacterial count in the liver to an average of 22 CFU compared to $>$1500 in control animals, a decrease of $>$67.5$\times$. This effect was identical to that of 20 (L100) and 5 (L25) mg/kg of levofloxacin, a potent fluoroquinolone, which had a similar effect to Pin2[G] in reducing the *S.* Typhimurium liver burden. Intermediate or lower concentrations of levofloxacin were not tested to minimize the numbers of animals used in experiments. Conversely, treatment with 0.56 mg/kg of FA1 was ineffective, since $>$1500 CFU were recovered.

Cytokine release and cytotoxicity in HBE and mouse RAW264.7 cell lines. Cationic amphipathic peptides can have immunomodulatory activities, including an ability to induce chemokines that can attract immune cells to the site of infection and anti-inflammatory activity in suppressing proinflammatory cytokines induced in re-

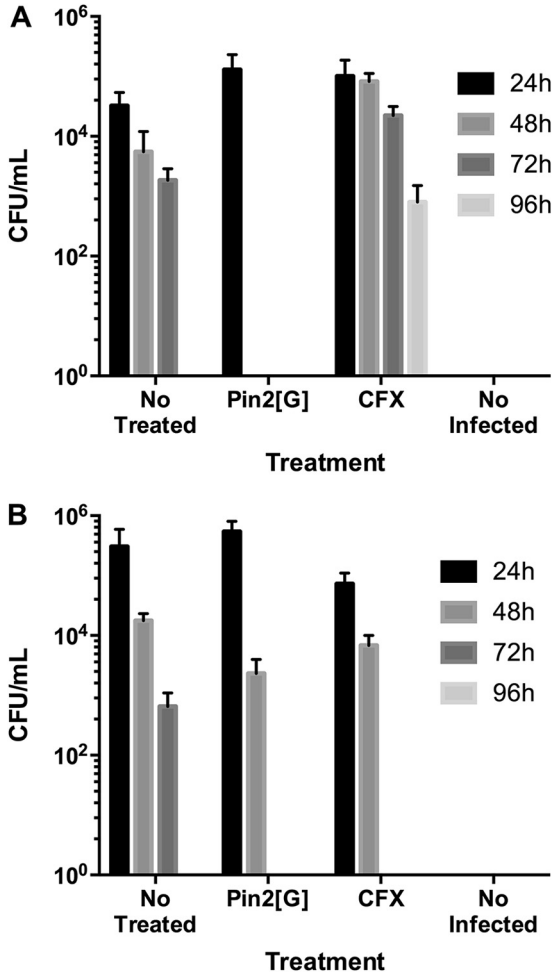

FIG 1 Bacterial counts of *S. aureus* UPD13 (A) and *P. aeruginosa* UPD3 (B) isolated from infected rabbit wounds after treatment with Pin2[G]. After infection, different treatments were applied to each wound at 24-h intervals for 4 days. No treated, wounds infected but not treated; Pin2[G], wounds infected and treated with 0.5 and 2 μg of Pin2[G] for *S. aureus* and *P. aeruginosa*, respectively; CFX, wounds infected and treated with 5 μg of ceftriaxone; No infected, wound without infection treated with PBS. Sample size $n = 2$; limit of detection, 100 CFU. Graph shows mean values and the error bars indicate standard deviations.

sponse to Toll-like receptor (TLR) agonists; these activities can promote protection of animals against *S. aureus* and *P. aeruginosa* infections (9, 10). First, we evaluated immunomodulatory activities in the human bronchial epithelial (HBE) cell line. Pin2[G] induced a dose-dependent increase of up to 3-fold in both proinflammatory cytokine IL-6 and chemokine IL-8 (Fig. 5A and C). Conversely, the presence of polyinosinic-polycytidylic acid (pI:C) increased these cytokines by 6.3- and 13-fold, respectively (Fig. 5B and D). The presence of Pin2[G] reduced pI:C-induced IL-6 and IL-8 by 55 to 68% and 59 to 68%, respectively (Fig. 5B and D). The cytokine/chemokine-inducing activity was more potent than that of control immunomodulatory peptide IDR-1018, while the anti-inflammatory effect in suppressing pI:C-induced cytokines was less than that of IDR-1018. FA1, on the other hand, was not effective at either activity.

We also examined analogous activities in the mouse monocytic cells line Raw264.7 (Fig. 6). In this case, Pin2[G] led to an upregulation of chemokine MCP-1 by $>$4-fold, while FA1 and IDR1-018 were much less active at the same concentrations. Conversely, lipopolysaccharide (LPS) increased proinflammatory TNF-α production to up to 2.4 ng/ml, while Pin2[G] led to a 4-fold decrease. Again, IDR-1018 was more active than Pin2[G], while peptide FA1 was less active.

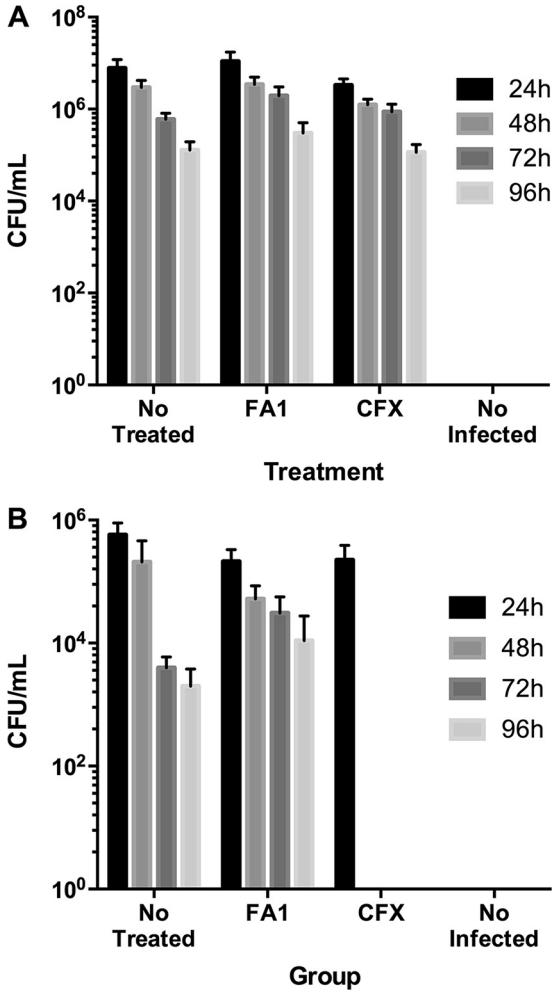

FIG 2 Bacterial counts of *S. aureus* UPD13 (A) and *P. aeruginosa* UPD3 (B) isolated from infected wounds after treatment with FA1. After infection, different treatments were applied to each wound at 24-h intervals for 4 days. No treated, wounds infected but not treated; FA1, both wounds were infected and treated with 2 μg of FA1; CFX, wounds infected and treated with 5 μg of ceftriaxone; No infected, wound without infection treated with PBS. Sample size $n = 2$; limit of detection, 100 CFU. Graph shows mean values and the error bars indicate standard deviations.

In agreement with the results of hemolysis assays, FA1 had lower cytotoxicity after 24 h treatment of both HBE and Raw cells compared to that of Pin2[G] (Fig. 7). Pin2[G] was indeed quite toxic in the concentration range evaluated (Fig. 7).

DISCUSSION

Antimicrobial resistance induced by the misuse of antibiotics is a problem impacting on the treatment of clinical infectious diseases. In contrast to conventional antibiotics, antimicrobial peptides (AMPs) have certain advantages, including activity against most antibiotic-resistant pathogens and a low propensity for resistance development, important characteristics for the control of multiresistant Gram-positive and Gram-negative bacteria. Their broad-spectrum activity and effective bactericidal activity makes them an interesting alternative for controlling bacterial infections (11). To date, >3,000 unique antimicrobial peptides, most of them host defense peptides, have been listed in the Antimicrobial Peptide Database (APD; http://aps.unmc.edu/AP/main.php). However, only few of them have been actually tested against multiresistant bacterial infections in animal models (12).

Here, we targeted diabetic foot ulcer pathogens, including *S. aureus* (incidence ~43%) and *P. aeruginosa* (incidence ~7 to 33%) (12). Both species have developed

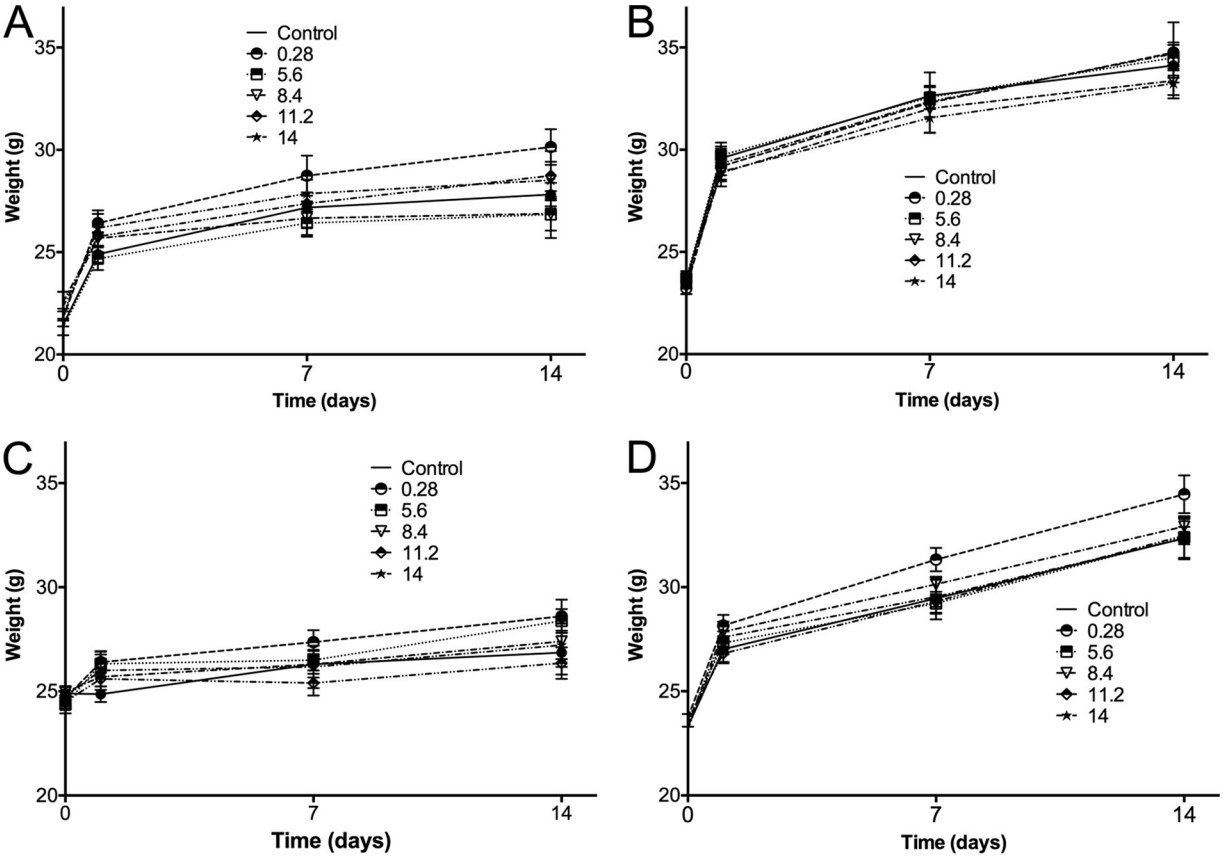

FIG 3 Weight of mice treated with either Pin2[G] or FA1. (A) Female mice treated with Pin2[G]. (B) Male mice treated with Pin2[G]. (C) Female mice treated with FA1. (D) Male mice treated with FA1.

substantial resistance to antibiotics and are important in the pathology of the diabetic foot ulcer. Therefore, the identification of novel bactericidal molecules that target such organisms is needed and earlier clinical studies suggested the potential of antimicrobial peptides against diabetic foot ulcers (13). Here, we tested the activity of two scorpion-derived HDPs against clinical isolates from diabetic foot ulcers (*S. aureus* UPD13 and *P. aeruginosa* UPD3). Pin2[G] was more effective at inhibiting the growth of Gram-positive *S. aureus* UPD13 (MIC = 7.5 μg/ml) than Gram-negative bacteria *P. aeruginosa* UPD3 (MIC = 60 μg/ml) and *S. enterica* serovar Typhimurium (MIC = 150 μg/ml). Relative to conventional antibiotics to which these organisms are susceptible, these activities, however, were in fact quite weak. Conversely, FA1 showed no antibacterial activity against *S. aureus* UPD13 (MIC >250 μg/ml) and quite weak antimicrobial activity against *P. aeruginosa* UPD3 and *S. enterica* serovar Typhimurium (MIC = 50 and 35 μg/ml, respectively). The activity of Pin2[G] was quite good against *S. aureus* UPD13, which was very resistant to five of the eight conventional antibiotics tested here, and this

TABLE 2 *Salmonella* Typhimurium ATCC 14028 CFU in the liver after treatments

	Bacterial counts[a]				
Mouse	Control	L100	L25	FA1	Pin2[G]
1	>1500	25	17	>1500	44
2	>1500	4	44	>1500	18
3	>1500	39	43	>1500	44
4	>1500	43	6	>1500	1
5	>1500	25	3	>1500	4

[a]Control, PBS alone; L100, levofloxacin 20 mg/kg; L25, levofloxacin 5 mg/kg; FA1, 0.56 mg/kg; Pin2[G], 0.56 mg/kg.

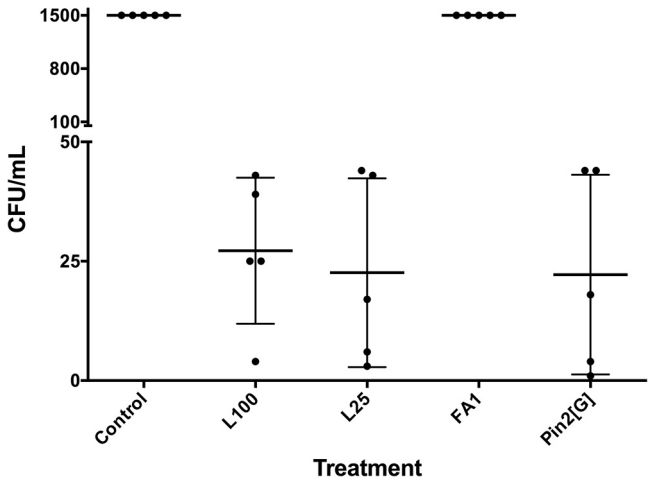

FIG 4 Bacterial counts of *Salmonella* Typhimurium ATCC 14028 from liver after treatment with Pin2[G] or FA1. After infection, different treatments were applied to each group of mice at 24-h intervals for 7 days. Control, PBS alone; L100, levofloxacin 20 mg/kg; L25, levofloxacin 5 mg/kg; FA1, 0.56 mg/kg; Pin2[G], 0.56 mg/kg. The geometric means are indicated by a horizontal bar.

peptide was approximately as effective as pexiganan/magainin (14), which has demonstrated activity in human diabetic foot ulcer patients (13), suggesting its potential. It seems likely that the amount of individual peptides that associate with different bacteria was influential in determining their relative activity due to the different physicochemical properties and structures (Fig. 8) of the peptides (15), which probably impacted on the complex mechanisms of action of such peptides (16). The *in vitro* results stimulated us to investigate the *in vivo* activities of these peptides.

Our animal model data showed that Pin2[G] inhibited the growth of *S. aureus* UPD13 in cutaneous infections at 48 h of treatment with 0.5 μg/dose, while ceftriaxone showed no major effect up to 72 h of treatment. Similarly, treatment of cutaneous *P. aeruginosa* UPD3 infections indicated that Pin2[G] eliminated this bacterium by 72 h with 2 μg/dose, while ceftriaxone showed the same result at 5 μg/dose. These results indicate that Pin2[G] was effective in the treatment of skin infections caused by these diabetic foot ulcer pathogens. Unfortunately, treatment with FA1, despite its superior MICs, did not show efficacy against the same bacteria. Similarly, FA1 was ineffective in treating a systemic enterohepatic *Salmonella* infection in mice. This lack of activity was consistent with previous reports that FA1 is degraded in human blood plasma, and likely by bacterial enzymes from *S. aureus* and *P. aeruginosa* (15), but may also reflect in part the weak antibiotic and immunomodulatory activities of this peptide.

As with the cutaneous infections, Pin2[G] at the very low dose of 0.56 mg/kg showed efficacy in decreasing *S. enterica* serovar Typhimurium systemic infection after 7 days of treatment, and this peptide was as effective as the positive control levofloxacin at 5 and 20 mg/kg. Overall, these results suggested that Pin2[G] was effective in controlling cutaneous infections caused by *P. aeruginosa* UPD3 and *S. aureus* UPD13 (isolates from diabetic foot ulcer) as well as systemic infections caused by *S. enterica* serovar Typhimurium.

Animal venoms have been demonstrated to be a valuable source of antimicrobial compounds with activities against multiresistant bacteria (17). However, many of these antimicrobials frequently present considerable cytotoxic effects against mammal cells which limits their clinical potential (17, 18). Although numerous approaches have been used to overcome this problem, such as specific amino acid substitutions of hydrophobic residues (19, 20), construction of hybrid peptides (19), physicochemical-guided rational design strategies (21), etc., *in vitro* cytotoxicity is generally measured in PBS, saline, or in tissue culture medium at low serum concentrations, maximizing the interaction between tested peptides and cells. This scenario is far from representing *in*

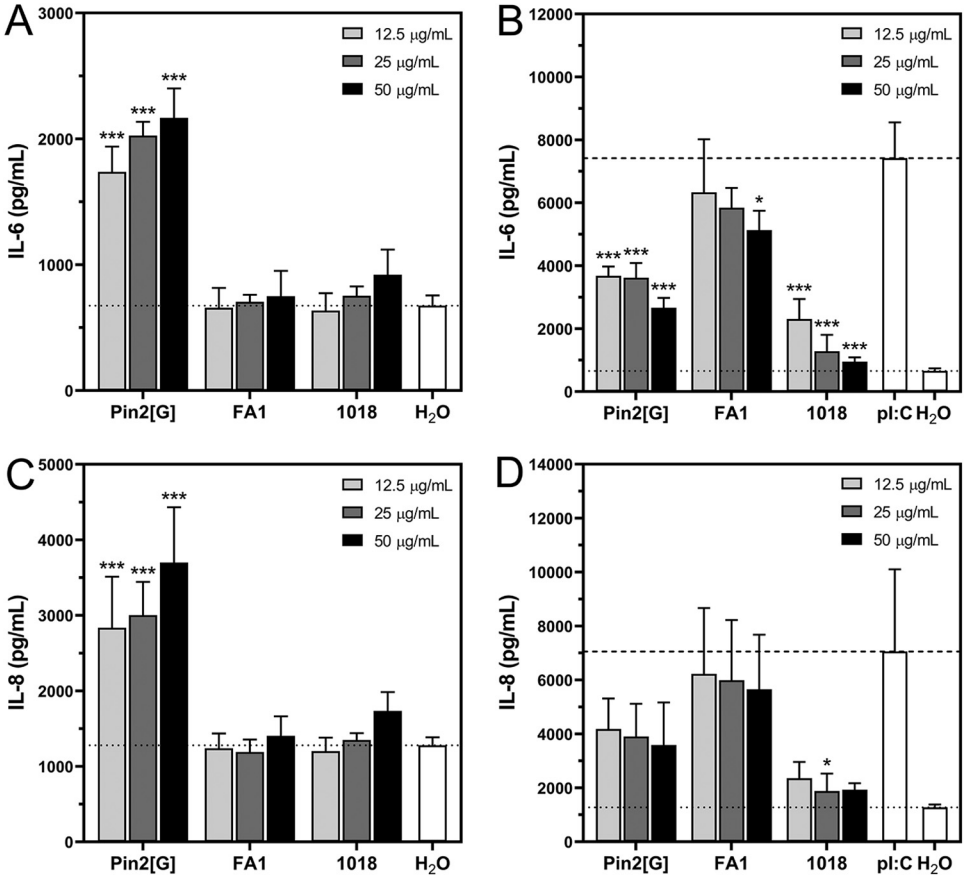

FIG 5 IL-6 and IL-8 production by HBE cells. Peptide-mediated induction and suppression of proinflammatory cytokines were evaluated by ELISA. Dose-response experiments were performed using Pin2[G], FA1, peptide IDR-1018 (as positive control), or water (as negative control) on unstimulated (A and C) and pI:C-stimulated HBE cells (B and D). Mean ± standard deviation of at least three biological replicates is shown. For reference, the basal production of each cytokine is represented by dotted lines, whereas the dashed lines represent the cytokine production by pI:C-stimulated cells treated only with water. Asterisks denote significant differences between treatment means compared with water (unstimulated cells) or pI:C (stimulated cells): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

vivo conditions, as further suggested here. Thus, in our study, Pin2[G] was highly hemolytic against human red blood cells (IC_{50} 1.2 μg/ml; Table 1), as well as demonstrating high cytotoxicity against human and mouse cell lines (Fig. 7). In contrast, when Pin2[G] was administered topically at 33-fold the hemolytic IC_{50} value onto the wounded skin of rabbits, no significant toxicity was observed. The same occurred when this peptide was intravenously administered to mice, where the only signs of moderate systemic toxicity were observed in the first few hours at a high dose (14 mg/kg, approximately 1,500- to 2,000-fold the hemolytic IC_{50} value). This lack of *in vivo* toxicity was consistent with the results of Torres et al. (21), who tested the *in vivo* activities of some synthetic analogs of the toxic wasp-derived polybia-CP using a scarification mouse model.

Conversely FA1, despite showing no hemolytic or cytotoxic activity *in vitro*, did cause signs of systemic toxicity in some mice injected with the peptide, and one mouse died shortly after administration. Overall these data indicate that the *in vitro* cytotoxicity assays currently used to characterize the activity of antimicrobial peptides may not reflect the outcome observed in the more complex scenario of *in vivo* conditions. Furthermore, Kumar et al. (22) clearly showed how cytotoxicity for such peptides can be mitigated by formulation. Therefore, peptides should not be discarded from further investigation if they fail to show low cytotoxic activity under lab conditions.

The high level of chemokine and cytokine production observed in Pin2[G]-treated

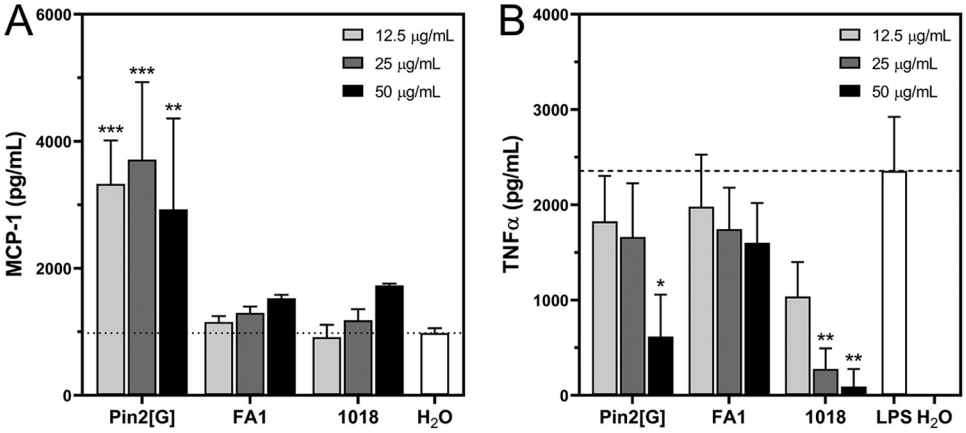

FIG 6 MCP-1 and TNF-α production by mouse Raw264.7 cells. Peptide-mediated induction of the MCP-1 chemokine and suppression of the TNF-α proinflammatory cytokine was evaluated by ELISA. Dose-response experiments were performed using Pin2[G], FA1, peptide IDR-1018 (as positive control), or water (as negative control) on unstimulated (A) and LPS-stimulated Raw264.7 cells (B). For reference, the basal production of MCP-1 cytokine is represented by a dotted line, whereas the dashed line represents the TNF-α production by LPS-stimulated cells treated only with water. Mean ± standard deviation of at least three biological replicates is shown. Asterisks denote significant differences between treatment means compared with water (unstimulated cells) or LPS (stimulated cells): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.00$.

unstimulated HBE (Fig. 6A and C) and Raw264.7 cells (Fig. 7A) was due either to stimulation of cells prior to cytotoxicity or a response to peptide-induced cellular damage. We favor the former explanation, since both of the characteristic features of HDP immunomodulatory activity were observed, namely, proinflammatory induction of specific cytokines and anti-inflammatory suppression of pI:C/LPS-induced cytokines. Thus, while we conclude that immunomodulatory activity might explain the strong performance of this peptide in animal infection models, further studies are needed to more profoundly understand the mechanism of action of Pin2[G] *in vivo*. Critically, FA1, which was not at all efficacious in animal models, had virtually no immunomodulatory activities.

As part of their antimicrobial mechanism of action, AMPs must first interact with bacterial surfaces. This interaction is initially mediated by electrostatic interaction between cationic peptides and the anionic surfaces of bacteria, including those of membranes. When the peptide reaches the cytoplasmic membrane, it first aligns

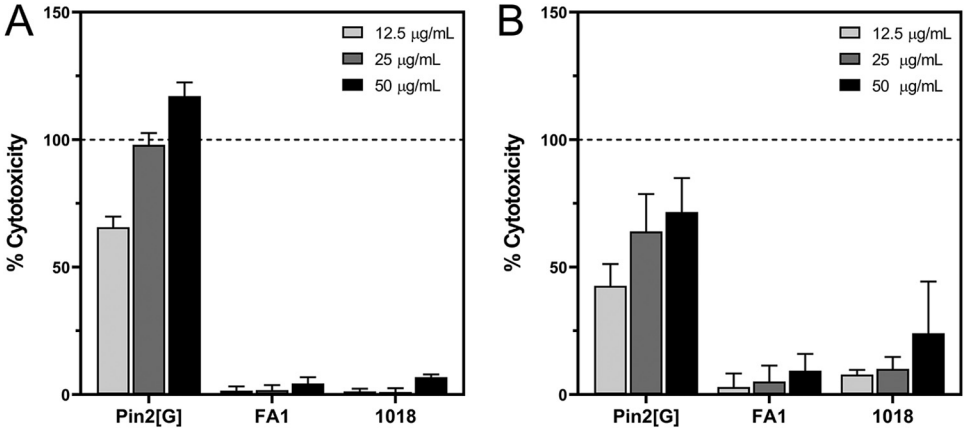

FIG 7 Peptide-induced cytotoxicity. The cytotoxic activities of peptides were evaluated against HBE (A) and mouse Raw264.7 cells (B) using the LDH release assay. Dose-response experiments were performed using three peptide concentrations. Data are shown as percent cytotoxicity with respect to the positive control of damage (2% Triton X-100). For clarity, 100% cytotoxicity is represented with dashed lines in both figures. Mean ± standard deviation of at least three biological replicates is shown.

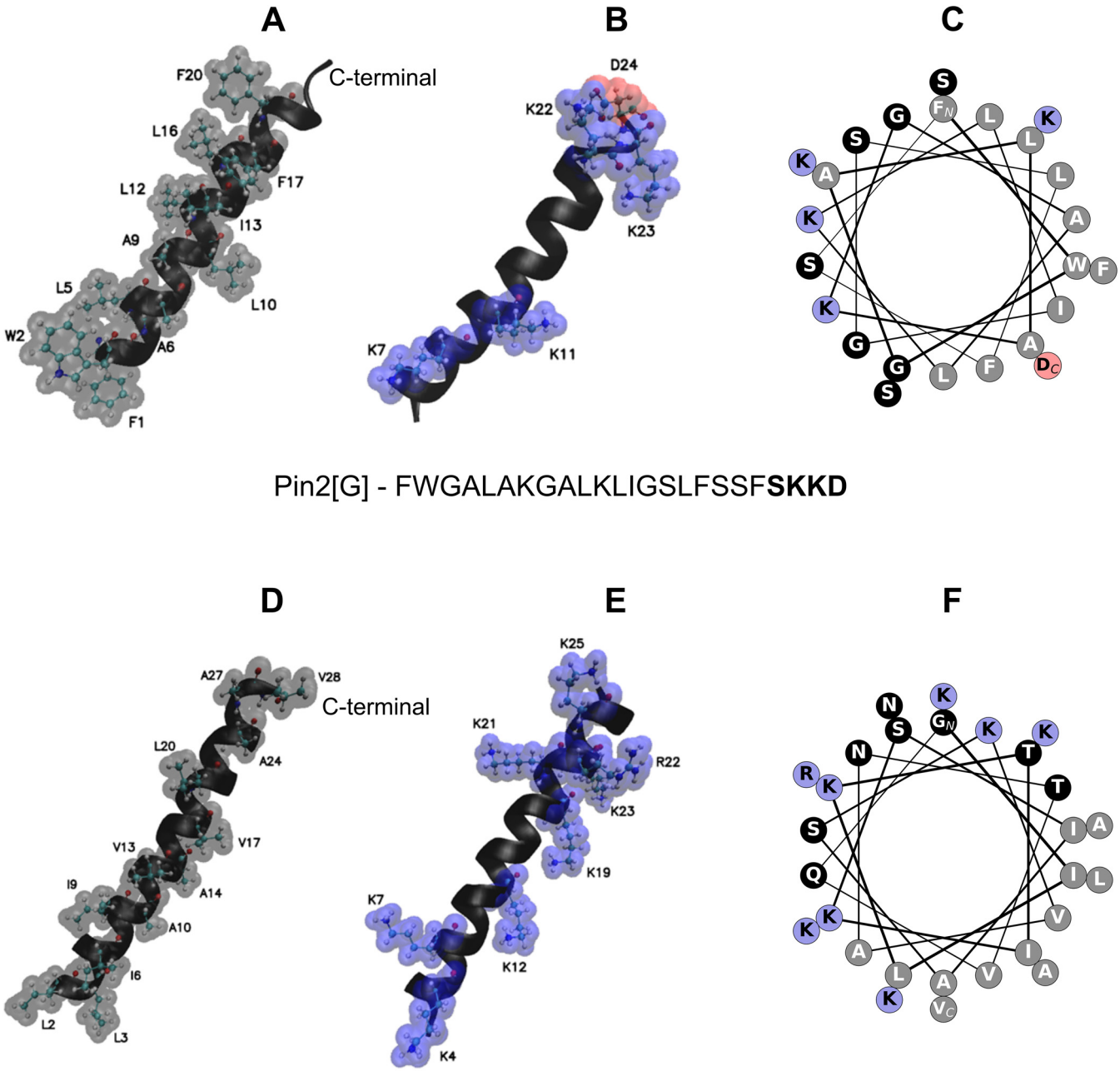

FIG 8 Structural models and primary structure of Pin2[G] and FA1. The models are based on secondary structure predictions using PDB, and also on experimental data from Sánchez et al. (24) and Rodriguez et al. (20). Pin2[G]: hydrophobic (A), cationic/anionic residues (B), and helical wheel projection (C). FA1: hydrophobic (D), cationic/anionic residues (E), and helical wheel projection (F).

electrostatically with the surface and then the hydrophobic domains of AMPs penetrate into the hydrophobic fatty acyl chains of phospholipids, influencing both membrane permeability and membrane-associated targets and/or, upon translocation, cytoplasmic targets (16). Therefore, the balance between hydrophobic and cationic residues in the peptides is important in determining their antimicrobial activity (23).

Pin2[G] and FA1 are cationic peptides with a considerable number of hydrophobic and hydrophilic amino acids and, although they have different size and residue composition, structural models suggest that they can both form amphipathic alpha-helical structures in

solution (20, 24) (Fig. 8). Fluorescence experiments with tagged Pin2[G] and FA1 have shown that both peptides interact with the bacterial membrane and are able to penetrate to the cytoplasm (unpublished data), suggesting they can also act on cytoplasmic targets. An example of an AMP that affects intracellular targets is buforin II, a well-studied amphibian alpha-helical peptide that penetrates the cell and interacts with bacterial DNA and RNA (25). The specificity of buforin II depends on its C-terminal region (-LLRK) (26), and, similarly to this peptide, FA1 and Pin2[G] have conserved basic C termini from positions 20 to 23 (-LKRK-) and 21 to 24 (-SKKD-), respectively. Other important factors to consider that would determine the biological activities of Pin2[G] and FA1 are the net charge and distribution of their basic residues. In this regard, increasing the charge of AMPs improves their antimicrobial activity, but beyond a certain limit an opposite effect can often be observed. For example, increasing, from +3 to +5, the net charge of well-studied amphibian alpha-helical magainin-2, led to an improvement of its antimicrobial effect versus both Gram-positive and Gram-negative bacteria. However, its antimicrobial potency decreased with greater increases in peptide charge (to +6 or +7), and the resultant peptides became more cytotoxic against human red blood cells (27). Based on this precedent, it might be speculated that FA1 (+8) would be more cytotoxic than Pin2[G] (+3), but in fact this did not occur. In contrast, Pin2[G] has a hinge region (Gly14) near the middle of its sequence that might also contribute to its *in vitro* cytotoxicity, as reported for other peptides (20). Regarding *in vivo* activity, it is worth considering our previous data (15) showing poor recovery of FA1 from plasma, which might reflect binding to plasma proteins and/or the lower stability of FA1 in solution relative to Pin2[G]. Thus, the differences between Pin2[G] and FA1 in net charge and distribution of hydrophobic residues, as well as interactions with other molecules *in vivo*, might explain their distinct antimicrobial performances under *in vitro* and *in vivo* conditions.

Conversely, invertebrate HDPs can also display immunomodulatory properties, such as the induction of cytokines and chemokines or suppression of proinflammatory mediators induced by bacterial signatures such as lipopolysaccharide, among other properties, which contribute to the overall anti-infective performance of these peptides against bacterial infections *in vivo* (28). The majority of these immunomodulatory effects are mediated by the direct or indirect interaction with cell surface or intracellular receptors or targets, with several molecules proposed as cognate binding partners for HDPs (29). The host cellular response to HDPs is a highly complex process that involves multiple signaling pathways, and varies depending on the cell type, peptide sequence, and inflammatory stimuli that are present (28). To investigate whether the structure and physicochemical characteristics of both peptides Pin2[G] and FA1 contribute to their immunomodulatory properties, we performed preliminary *ab initio* modeling and molecular docking with the CC-chemokine receptor type 2 (CCR2). This was based on binding sites that the chemokine receptor has for human beta-defensin 6 (30). In our preliminary modeling (data not shown), it was observed that there was certain electrostatic affinity of CCR2 to both peptides, consistent with previous observations by Shi et al. (31), who reported that all CCR2 ligands are cationic molecules with charges from +5 to +9. Therefore, among others, CCR2 receptors in Raw264.7 and HBE cells may be induced to activate signaling pathways (32, 33). Likewise, molecular docking has been successfully used to predict the interaction of HDPs with the TLR-4/MD2 complex (16). In this regard, the *ab initio* modeling of Pin2[G] and molecular docking experiments with known receptors involved in the cellular response to HDPs might highlight key residues for the interaction of these peptides with host receptors. Pin2[G] and, to much lesser extent, FA1 were able to suppress proinflammatory cytokines from stimulated mammalian cells, which suggests that these peptides interfere in signaling responses by proinflammatory pI:C and LPS. Such docking studies will enable advanced predictions that can subsequently be confirmed experimentally by generating and testing the activity of the specific mutants. We also suggest that such studies of the immunomodulatory properties of peptides might involve fragments derived from these peptides to increase our understanding of structure-activity relationships and identify any functional domains. Thus, for future studies it will be interesting to pursue this type of

experiment, guided by observations of changes in *in vivo* cytokine/chemokine release in mouse blood samples after treatment with such peptides.

In conclusion, we have shown that a scorpion-derived synthetic peptide named Pin2[G] was effective versus cutaneous infections caused by *P. aeruginosa* and *S. aureus*, as well as hepatic infections caused by *S. enterica* serovar Typhimurium, despite its weak *in vitro* MICs and high *in vitro* cytotoxicity. Our data indicate that both *in vitro* and *in vivo* experimental approaches are necessary to characterize the potential of anti-infective peptides. These results may warrant further consideration for Pin2[G] and other antimicrobial peptides as leads for cutaneous and perhaps systemic infections.

MATERIALS AND METHODS

Compliance with ethical standards. Animal experimental procedures were in accordance with international recommendations and the guidelines of the Good Experimental Practices, under the supervision of the Ethical and Animal Welfare Committee of the UNIPREC (https://quimica.unam.mx/investigacion/servicios-para-la-investigacion/uniprec/). Animals were housed, handled, and cared for in accordance with the official Mexican standards for the care and use of laboratory animals (no. NOM-062-ZOO-1999).

Biologicals. *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, and *S. enterica* serovar Typhimurium ATCC 14028 were purchased directly from the American Type Culture Collection through The Global Bioresource Center by UNAM. *S. aureus* UPD13 and *P. aeruginosa* UPD3 were isolated from diabetic foot ulcers at a local hospital in Cuernavaca, Morelos, Mexico. The murine macrophage cell line RAW 264.7 was also obtained from the American Type Culture Collection (ATCC TIB-71). Simian virus 40-transformed immortalized human bronchial epithelial (HBE) cells (16HBE14o-) were kindly donated by D. Gruenert (University of California at San Francisco) (34). The HDPs Pin2[G] (FWGALAKGALKLIGSLFSSFSKKD) and FA1 (GILKTIKSIASKVANTVQKLKRKAKNAV) were chemically synthesized using the Fmoc method (15). The innate defense regulator (IDR)-1018 peptide (VRLIVAVRIWRR-NH_2) was chemically synthesized by CPC Scientific Inc. (Sunnyvale, CA) and obtained at >95% purity. The commercial antibiotics were donated from Laboratorios Liomont SA de CV (Mexico City, Mexico).

Animals. New Zealand rabbits and Hsd:ICR (CD-1) mice were purchased from Harlan Sprague Dawley, Inc. (Mexico City, Mexico). Management of animals followed the recommendations of the *Guide for the Care and Use of Laboratory Animals* (35), and in accordance with the official Mexican standards for the care and use of laboratory animals (no. NOM-062-ZOO-1999) (36). Mice, 6 to 8 weeks old, 20 to 25 g, and either female or male were acclimated to the laboratory for 6 days prior to the start of infections. Only animals in good health were selected for use. Animals were reared on standard lab diet (for mice Teklad 2018SX, Envigo, with wheat, corn and soybean; for rabbits Teklad 2031, Envigo, with dehydrated alfalfa meal, ground wheat, wheat bran, ground oats) and tap water *ad libitum*, and maintained in an air-conditioned room at 19 to 25°C, with a relative humidity of 40 to 70%, a 12-h light (7:00 to 19:00)/dark (19:00 to 7:00) cycle, and ventilation of 15 to 21 air changes/h.

Antimicrobial activity. The MIC was determined using the broth microdilution assay in accordance to the procedures from the Clinical and Laboratory Standards Institute (35). For broth microdilution assays, bacteria were cultured in Mueller-Hinton broth (MHB) at 37°C until an endpoint of between of 0.08 and 0.13 units of absorbance at 625 nm (ca. 1 to 2×10^8 CFU/ml) and diluted 1:100 in MHB. Fifty microliters of each bacterial suspension was dispensed into each well of a 96-well microtiter Costar culture plate (Sigma-Aldrich, St. Louis, MO, USA) containing 50 μl of MHB with a 2-fold dilution series containing varied concentrations of the HDPs (50, 25, 12.5, and 6.2 μg/ml) or antibiotics (50, 25, 12.5, 3.2, and 1.6 μg/ml) used. The minimal concentration preventing bacterial growth after 18 h of incubation at 37°C using a Sunrise plate reader from Tecan Group Ltd. (San Jose, CA, USA) was recorded as the MIC.

Hemolytic assays. Human red blood (hRB) cells (O, Rh-positive) were collected from a healthy male volunteer who signed an informed consent to use his blood for hemolytic assays. The experimental protocol to study hemolysis was approved by the Bioethics Committee of the Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM). Hemolytic activities were determined by incubating suspensions of hRB cells in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) with serial dilutions (from 0.5 to 256 μg/ml) of either Pin2[G] or FA1 and measuring hemoglobin liberation by absorbance at 570 nm after 1 h at room temperature. Each assay was performed in triplicate, and data were expressed as mean ± SD. Percentage of hemolysis was calculated using the formula: % hemolysis = 100 (Abs/peptide-Abs/PBS)/(Abs/H_2O-Abs/PBS). The peptide concentration that caused 50% hemolysis of the human erythrocytes (IC_{50}) was obtained by fitting data to a logistical sigmoidal equation using the software package GraphPad Prism, v. 4.0 (CA, USA).

Dermal irritation to rabbits. Acute skin irritation tests were performed to determine the ability of Pin2[G] and FA1 to cause erythema or edema in New Zealand albino rabbits by applying different concentrations of peptide (up to 50 μg/ml). Treatments were applied on the shaved backs of rabbits at different time intervals, with subsequent monitoring for secondary effects over 14 days. The procedure was performed according to the United States Environmental Protection Agency guidelines 870.2500 (37). Briefly, each peptide was dissolved in PBS at 50 μg/ml. Only rabbits with healthy, intact skin were used (17 weeks of age, in the range of 3 to 3.6 kg in body weight). An area of approximately 10 to 15 cm on the back of each rabbit was made free of fur using electric clippers and an electric shaver 24 h prior to testing. The sample solution (0.2 ml) was applied on the skin and covered with a gauze patch, which was held in place with nonirritating elastic bandage. In an initial test, using only one rabbit, no dermal

reactions were observed at 3 min or at 1 or 4 h after the patch was removed. We then proceeded to repeat the test with another two rabbits to confirm the initial findings ($n = 3$). The prolonged application of the peptides was also examined in a dermal toxicity test, in which different concentrations of peptides up to 10 μg/dose were used and their effect recorded after 24 h of exposure (37). The skin adjacent to the test sites was considered the negative control.

Toxicity in mice. Toxicity of either Pin2[G] or FA1 was performed on Hsd:ICR (CD-1) mice in 6 groups ($n = 5$) of males and 6 groups ($n = 5$) of females, i.e., 60 mice for each peptide. All groups were intravenously administered with peptides in 150 μl of PBS at doses of 0, 0.28, 5.6, 8.4, 11.2, or 14 mg/kg and maintained for 7 days. Mice were observed individually during the first 30 min after injection, and periodically during the first 24 h (with special attention during the first 4 h) and later every day, for a total of 14 days. For individual monitoring records, a format for the evaluation of clinical signs of acute toxicity was used, where clinical signs such as mortality, locomotive activity, sensitivity to sound, abnormal tail appearance, exploratory behavior, aggressive behavior, seizures, muscle tone in extremities, somatic response, prostration, tremors, lacrimation, ptosis, defecation, micturition, changes in respiration, discharges from nose, cyanosis, and piloerection were monitored and recorded (38). The weight of each animal was recorded on day 0 (reception of the animals) and days 1 (first day of administration), 7, and 14 postadministration, and they were also recorded according to the standard procedure of operation for toxicity (37).

Animal wound infection. Animal experimental procedures were performed in accordance with international recommendations and the guidelines of the Good Experimental Practices, under the supervision of the Ethical and Animal Welfare Committee of the "Unidad de Investigación Preclínica" UNIPREC (https://quimica.unam.mx/investigacion/servicios-para-la-investigacion/uniprec/). New Zealand albino rabbits ($n = 4$) were separated according to the treatment to be received. One day before the treatment, the back of New Zealand rabbits (3 to 4 kg) was shaved with an electric razor (Wahl Pet Clipper, model pcmc-2, Sterling, IL, USA) and disinfected with 70% ethanol. The next day, animals were anesthetized intramuscularly with 50 mg/kg of ketamine and 4 mg/kg of 10% xylazine (PiSA Agropecuaria Inc., Mexico). Subsequently, four areas of 2×2 cm were outlined on the back of each rabbit and disinfected with iodopovidone (Pierre Fabre Farma, Mexico). A circular section of skin with a diameter of approximately 1 cm^2 of area and approximately 0.5 to 1 mm deep was removed with a scalpel. Wounds were initially biopsied with a sterile swab to assess viable bacterial count by serial dilution (CFU), to confirm the sterility of the area, using the method of York et al. (39). Bacterial application to the wounds utilized bacteria grown overnight in 3 ml of Luria Bertani (LB) broth from which 1 ml was taken and centrifuged at 14,000 rpm for 5 min. The cell pellet was resuspended in 50 μl of fresh LB broth and then applied on 3 of the wounds/rabbit. The fourth rabbit wound was not infected to serve as a negative control. After air drying the inoculum, wounds were covered with sterile gauze and this process was repeated at 12 h to ensure an infection. After 24 h of the bacterial inoculation, the infected wounds were treated with 50 μl of either distilled water, Pin2[G], or FA1, or 50 μl containing 5 μg/dose of ceftriaxone, while the uninfected wound also received distilled water. Ceftriaxone was used as a positive control as it is often used to treat incisional surgical site infections (40). Based on the *in vitro* results, rabbit wounds infected with *S. aureus* UPD13 were treated with 0.5 μg/dose of Pin2[G] or 2 μg/dose of FA1, and those infected with *P. aeruginosa* UPD3 with 2 μg/dose of Pin2[G] or FA1, respectively. Antimicrobial treatments were performed every 24 h for 4 days. Prior to each treatment and at the end of the experiment, wounds were swabbed to assess bacterial growth in the wounds.

***In vivo* challenge experiments with *Salmonella enterica* serovar Typhimurium.** An overnight culture of *S. enterica* serovar Typhimurium (ATCC 14028) in LB broth (Sigma) was centrifuged (3,500 $\times$ *g*, 15 min, 20°C) and washed twice with PBS, pH 7.4. Subsequently, Hsd:ICR (CD-1) mice were intragastrically challenged with 5×10^8 CFU/ml (300 μl in PBS) of *S. enterica*. Mice were divided into 5 groups according to the treatment received (5 animals per group). All mice were kept in positive-pressure cabinets and monitored for a week in the experimental isolation facilities at the UNIPREC. To test the anti-*Salmonella* activity of peptides, mice were treated intravenously at a dose of 0.56 mg/kg once daily for 7 days. At the end of infections, mice were sacrificed by cervical dislocation and the right liver lobes were homogenized in a Seward Stomacher 80 Biomaster (Seward) in 10 ml of PBS. Viable bacterial counts were assayed on pour plates of MacConkey agar as the growth medium.

Treatment of human bronchial epithelial cells. HBE cells were grown and treated as described previously (41). Briefly, cells in minimum essential medium (MEM) (Thermo Fisher, Gibco, Grand Island, NY, USA, number 11090081) supplemented with 10% fetal bovine serum (Thermo Fisher, Gibco, Canada, number 12483020) and 2 mM L-glutamine (Thermo Fisher, Gibco, Grand Island, NY, USA, number 25030081) (10% MEM) were seeded at 5×10^4 cells/well in a 96-well flat-bottom tissue culture-treated polystyrene plate (Costar, Kennebuk, ME, USA, number 3596). After overnight growth at 37°C with 5% CO_2, cells were rinsed and then incubated for 1 h in 100 μl of 1% MEM media. Subsequently 80 μl of 1% MEM, with or without the Toll-like receptor (TLR) 3 agonist polyinosinic-polycytidylic acid (pI:C) (InvivoGen, San Diego, CA), at a final concentration of 100 ng/ml, was added to the cells. The cells were treated with 20 μl of peptides (dissolved in water at 10$\times$ the desired final concentration) or water as the vehicle control. The HBE plates were incubated overnight using the same conditions as above and the following day (approximately 24 h later) supernatants were collected into 96-well polypropylene plates (Costar, Kennebuk, ME, USA, number 3879) for cytotoxicity assays and ELISA.

Treatment of Raw264.7 cells. Murine Raw264.7 monocytic cells were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher, Gibco, Grand Island, NY, USA, number 10313021) supplemented with 10% fetal bovine serum and 4 mM L-glutamine (10% DMEM). Cells (200 μl) were seeded at 2×10^4 cells/well in a 96-well flat-bottom tissue culture-treated polystyrene plate for 48

h at 37°C with 5% CO_2 (medium was refreshed after 24 h). After that, cells were rinsed with 10% DMEM, 100 μl of the medium was added, and the cells were treated as described above for the HBE cells. The TLR4 agonist *P. aeruginosa* PAO1 lipopolysaccharide (LPS), purified by the Darveau-Hancock method (42), was added to some wells at a final concentration of 100 ng/ml. The cells were treated with either 20 μl of peptides (dissolved in water at 10$\times$ the desired final concentration) or water as the vehicle control. The plates were incubated overnight at 37°C as above and the following day (approximately 24 h later), supernatants were collected into 96-well polypropylene plates for cytotoxicity assays and ELISAs.

Lactate dehydrogenase assay. The cytotoxicity of the peptides against HBE and Raw264.7 cells was evaluated using the Cytotoxicity Detection kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturers' instructions. The kit measures the enzyme activity of lactate dehydrogenase (LDH) released from damaged cells. Supernatants of cells treated with vehicle (water) and lysed by adding 2% Triton X-100 to the cells 1 h prior to collecting the sample supernatants served as the negative (N, 0% toxicity) and positive (P, 100% toxicity) controls, respectively. Absorbance at 490 nm was measured after 20 min and percent cytotoxicity was calculated using the following formula: % cytotoxicity = 100 $\times$ [(E $-$ N)/(P $-$ N)], where E denotes experimental absorbance of peptide-treated cell supernatants. LDH assays were carried out on at least 3 separate biological replicates for both HBE and Raw264.7 samples.

Enzyme-linked immunosorbent assays. Enzyme-linked immunosorbent assays (ELISA) kits from eBioscience Inc. (San Diego, CA, USA or Invitrogen (Carlsbad, CA, USA) were used to quantify the levels of monocyte chemotactic protein 1 (MCP-1), interleukin 8 (IL-8), and IL-6. All ELISAs were carried out on at least 3 separate biological replicates.

Three-dimensional structure prediction of FA1 and Pin2[G]. The three-dimensional structures of both FA1 and Pin2[G] were predicted using the iterative threading assembly refinement (I-TASSER) server (43, 44) and Pep fold server of RPBS, a web resource for structural bioinformatics (45). Additionally, structural data from Pin2 (46) were used to build the final structure of Pin2[G]. The RMSD of main chains (N, Cα, C, and O) were used to compare the models obtained from both servers. The final three-dimensional structures have the best RMSD values. Helical wheel projections were computed using the modlAMP package in Python (version 3.8) (47).

Statistical analysis. The least significant difference method was used to determine whether statistically significant differences occurred among the mean values obtained using the software package Prism 4 (GraphPad Prism, v. 4.0, CA, USA). In the *Salmonella* Typhimurium ATCC 14028 infection model, data were analyzed by nonparametric one-way of variance Mann–Whitney U-test (XLSTAT software: http://www.xlstat.com) and by the log rank test (GraphPad Prism, v. 4.0, CA, USA). Data from the cytokine release experiments were analyzed using Dunnett's multiple-comparison test, the Kruskal–Wallis nonparametric method, or Dunn's multiple-comparison test. *P* values less than 0.05 were considered statistically significant.

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We declare no conflicts of interest.

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